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Bacteriophage T4 Virion Baseplate Thymidylate Synthetase and Dihydrofolate Reductase

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Additional evidence is presented that both the phage T4D-induced thymidylate synthetase (gp *td*) and the T4D-induced dihydrofolate reductase (gp *frd*) are baseplate structural components. With regard to phage *td* it has been found that: (i) low levels of thymidylate synthetase activity were present in highly purified preparations of T4D ghost particles produced after infection with td^+, whereas particles produced after infection with td^- had no measurable enzymatic activity; (ii) a mutation of the T4D *td* gene from td^{ts} to td^+ simultaneously produced a heat-stable thymidylate synthetase enzyme and heat-stable phage particles (it should be noted that the phage baseplate structure determines heat lability); (iii) a recombinant of two T4D mutants constructed containing both td^{ts} and frd^{ts} genes produced particles whose physical properties indicate that these two molecules physically interact in the baseplate. With regard to phage *frd* it has been found that two spontaneous revertants each of two different T4D frd^{ts} mutants to frd^+ not only produced altered dihydrofolate reductases but also formed phage particles with heat sensitivities different from their parents. Properties of T4D particles produced after infection with parental T4D mutants presumed to have a deletion of the *td* gene and/or the *frd* gene indicate that these particles still retain some characteristics associated with the presence of both the *td* and the *frd* molecules. Furthermore, the particles produced by the deletion mutants have been found to be physically different from the parent particles.

Considerable genetic and biochemical evidence has been presented from several laboratories (4, 12, 15, 16, 19-21) that two early phage-induced enzymes, dihydrofolate reductase, called gp *frd* (25) (referred to earlier as gp *dfr* [12]), and thymidylate synthetase, called gp *td* (3, 11), were components of the T-even phage baseplate. However, since neither the known missense mutations (5) nor the known nonsense mutations (17) in these genes prevented phage formation, the gene products were classified by Wood and Revel (25) as nonessential components of the baseplate. This problem was complicated both by the failure to detect proteins corresponding to these enzymes upon polyacrylamide gel analysis of phage particles (10, 24) and by isolation by viable phage particles presumably containing deletions of the *td* and *frd* genes (7). Earlier work from Mathews' laboratory (20) and this laboratory (11, 16) indicated that these two gene products were not required as intact functioning enzymes, but that substantial portions of these polypeptides could play the required structural role. It should also be noted that phage baseplates contain an unusual form of folic acid, dihydropteroyl hexaglutamate, as a structural component, which is closely linked to the two enzyme molecules (15). This paper and the accompanying report by Mosher et al. (22), which deals largely with gp *frd* in phage particles, present additional chemical, genetic, and enzymatic data that these two proteins are present in all phage particles yet examined. The thymidylate synthetase and the dihydrofolate reductase molecules have been shown to be partially buried within the baseplate structure. Furthermore, these two reports point out several anomalies in the properties of the presumed T4D deletion mutants of the *frd* and *td* genes that suggest that the physical and genetic mapping of the deletion mutants may need reinterpretation.

MATERIALS AND METHODS

Preparation and purification of bacteriophage stocks and isolation of mutants. *Escherichia coli* bacteriophage stocks were grown, purified, and assayed by standard procedures (11, 12, 15). When used to measure thymidylate synthetase activity, the purified phage particles were osmotically shocked, and the ghost particles were further puri-

fied on D_2O-sucrose gradients (15). The T2L and T4D strains have been used in this laboratory for some time. The two T4D amber mutants, one in the *frd* gene (*frd*11) and one in gene 11 (N9s), were those described earlier (15). T4D *td*6, a missense mutation in the *td* gene, was obtained from D. H. Hall (9). The isolation and properties of a T4D td^{ts} mutant (strain 408) were described earlier (11). The two frd^{ts} mutants, originally obtained from D. H. Hall, P1 and C31, and their parent $T4D_0$ strain have also been described (5, 12). T4 phage bearing deletions presumed to be between genes 63 and 32, originally isolated by Homyk and Weil (7) and crossed by Homyk and Weil into a T4D strain called 1589, were obtained from C. Mathews. The T4D 1589 strain contained a deletion in the *r*II gene that was used as a DNA marker (7). These deletion strains are labeled by the names originally assigned by Homyk and Weil as *del*1, *del*7, and *del*9. The bacterial host strains used included *E. coli* B and three mutants of *E. coli* B affecting host dihydrofolate reductase activity, *E. coli* B TRIM 102 and TRIM 103 (12) and *E. coli* B RT 500, obtained from D. Baccanari (1). *E. coli* B201, which was td^- and grew adequately when the medium was supplemented with low concentrations of thymine, was obtained from C. Mathews. The standard *E. coli* su^+ strain used was CR63. *E. coli* OK305 was used to assay for the "white halo" plaques produced by frd^- and td^- phage strains (6).

The enzyme assays and other methods have all been described previously (12, 15). Standard reagents were obtained from commercial sources.

RESULTS

Thymidylate synthetase activity in phage ghost particles. Capco and Mathews (3) presented the original evidence that the phage-induced thymidylate synthetase molecule was a phage component. Later work from this laboratory indicated that this enzyme was a baseplate component (11). In both laboratories, early attempts to demonstrate thymidylate synthetase activity in phage particles were unsuccessful. Further efforts were now made with higher concentrations of phage ghost particles in the highly sensitive radioactive assay (18). The final concentration of phage ghosts used was about 10^{13}/ml in the reaction mixture. Those particles showing thymidylate synthetase activity released radioactivity from [^{3}H]dUMP linearly for 1 to 2 h, and the enzymatic rate was calculated per particle added (Table 1). Although the baseplate thymidylate synthetase is known to be partially covered by gene 11 and 12 proteins (11), assays of T4D ghost particles lacking the 11 and 12 proteins consistently showed less thymidylate synthetase activity than did preparations of whole T4D ghosts. Similar amounts of enzyme activity were found in preparations of T4D, T4D strain 1589, and T2L, and somewhat less was found in T4D *frd*11 ghosts. No activity could be detected in preparations of the missense *td* mutant T4D *td*6 or in the three T4D deletion mutants obtained by Homyk and Weil (7), which also do not induce thymidylate synthetase activity. The activity per particle of T4D (and T2L) is only a small fraction of the activity of a single thymidylate synthetase molecule, as calculated from the turnover number of the purified enzyme given by Capco et al. (2). Since this enzyme is presumed to be part of the complex baseplate structure, a variety of disruptive procedures were carried out in attempts to free the enzyme in a manner analogous to the way gentle urea or formamide treatment exposed dihydrofolate reductase activity in T4D ghosts or substructures (16). These procedures included pretreatment of the ghosts with 10^{-4} M reduced nicotinamide adenine dinucleotide phosphate (NADPH) (16), 0.02 M mercaptoethanol, cold 60 to 70% dimethyl sulfoxide, 0.01% cetyltrimethylammonium bromide, 67% glacial acetic acid, and sonic oscillation. None of these treatments liberated increased thymidylate synthetase activity. However, it should be pointed out that free T4 thymidylate synthetase is very unstable (3, 17) and is rapidly inactivated by denaturing agents. The observed enzymatic activities support the conclusion that the virion enzyme found in these preparations is a product of the phage *td* gene.

TABLE 1. *Thymidylate synthetase activity in preparations of phage ghost particles*

Ghost particle preparation	Activity[a] (mol/min per 10^{13} particles)
T2L	0.07 ± 0.01
T4D	0.16 ± 0.02
T4D *frd*11	0.03 ± 0.01
T4D *td*6	<0.01
T4D 1589	0.20 ± 0.03
T4D *del*1	<0.01
T4D *del*7	<.01
T4D *del*9	<.01
Purified T4D thymidylate synthetase (1 molecule)[b]	3.8

[a] Enzyme activity was measured by the method of Lomax and Greenberg (18) as modified earlier (12).
[b] Calculated from the report of Capco et al. (2).

Properties of a T4D td^{ts} revertant. It was reported earlier that two td^{ts} mutants of T4D produced both heat-labile thymidylate synthetase molecules and phage particles with increased heat sensitivities (11). Although these two td^{ts} mutants were backcrossed four times against wild-type T4D, they were isolated after mutagenesis, and the possibility existed that the mutagenesis was responsible for the production of another capsid protein that was heat

sensitive. A single plaque was picked of the most heat labile of these two mutants (strain 408), and, using this stock, a search was made for a wild-type td^+ revertant. This parent strain 408 mutant gave normal plaques on *E. coli* OK305 at 30°C but gave plaques with white halos at 43°C. Plating with *E. coli* B201 confirmed that the parent 408 was td^- at 43°C but was td^+ at 30°C. One plaque (out of 13,000 of strain 408 examined) was of the normal type at the high temperature and was also td^+ at 43°C on B201. This revertant, presumably due to a single spontaneous mutation, was isolated, and its properties were examined. The revertant not only produced a heat-stable thymidylate synthetase, as compared with parent strain 408, but also produced phage particles that were more heat stable than the 408 and even more heat stable than the original parent T4D particles (Fig. 1).

Preparation and properties of a T4D *frd*^{ts} *td*^{ts} mutant. Evidence from earlier work (11) had shown that the baseplate thymidylate synthetase could physically interact with the baseplate dihydrofolate reductase. This earlier work involved preparing and characterizing the properties of a double T4D mutant, using strain 408, a td^{ts} mutant, as one parent and an frd^{ts} mutant known as P1 (9) as the other parent. A second double mutant was now prepared by using strain 408 again as one parent but a different frd^{ts} mutant, known as C31, as the other parent. The procedure was as described earlier. A cross of the two mutants was made with strain 408 as the majority parent and C31, a T4D *far* mutant, which is also resistant to pyrimethamine (9), as the minority parent. The progeny of the cross were plated on bacteria on media containing pyrimethamine, and plaques were picked and tested until one recombinant was obtained that both produced pryimethamine-resistant plaques and gave the *td* white-halo plaque on OK305 only at the high temperature. The recombinant was also td^- on B201 at the high temperature. The heat sensitivity of this double mutant was compared to the majority parent 408, and Fig. 2 shows the typical results obtained in several experiments. As found previously, the heat sensitivity of both the 408 mutant and the recombinant was a

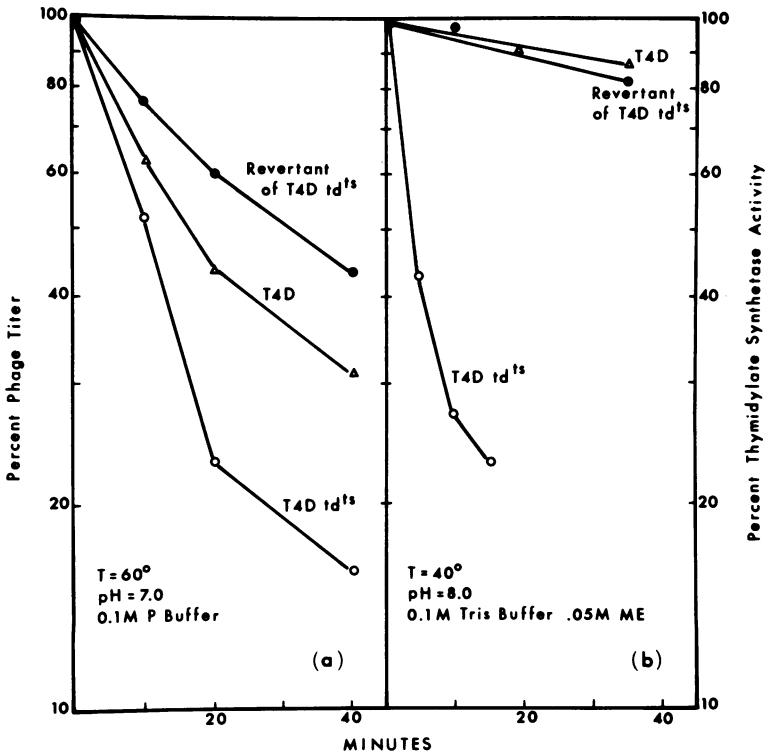

FIG. 1. *Heat sensitivity of various T4D particles and the T4D-induced thymidylate synthetases. (a) The phage particles were heat inactivated in the standard way at 60°C (11). (b) Extracts were made of E. coli B infected with the various types of phage. These extracts were heated at 40°C, and the activity of the remaining thymidylate synthetase was determined. Abbreviations: P, Phosphate; ME, mercaptoethanol.*

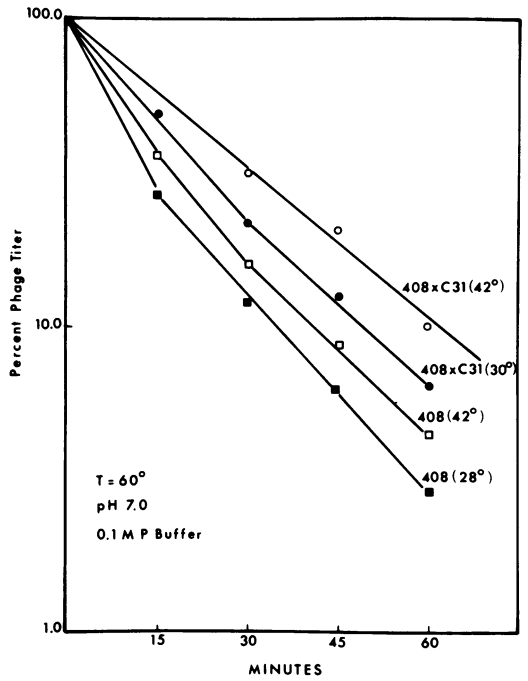

FIG. 2. *Heat sensitivity of T4D particles produced by recombination of T4D td^{ts} (408) and frd^{ts} (C31) mutants. The recombinant T4D, which is td^{ts} frd^{ts}, was prepared as described, with the td^{ts} as the majority parent. Phage stocks were grown either at 28 to 30°C or at 42°C, and the heat sensitivity at 60°C of the phage particles was determined in the usual way. P, Phosphate.*

function of the temperature of assembly (whether at 42 or at 30°C). Irrespective of the temperature of assembly, the double mutant T4D *td*^{ts}*frd*^{ts} was more heat resistant than the *td*^{ts} majority parent. These changes in heat sensitivity indicate again that the heat labilities of these two baseplate components are not directly additive. The results support the conclusion that these two baseplate components influence each other physically, either directly or indirectly.

Physical properties of revertants of T4D *frd*^{ts} to *frd*$^{+}$. The original *frd*^{ts} phage particles were isolated as spontaneous mutants by Johnson and Hall (9) and were first characterized by their ability to form plaques on bacteria in the presence of anti-dihydrofolate reductase compounds, such as pyrimethamine. Johnson and Hall (9) showed that two of these drug-resistant mutants produced altered enzymes that were both drug resistant and heat labile. Later, Kozloff et al. (12) additionally characterized these T4D *frd*^{ts} mutants and showed that they produced heat-labile phage particles. A search was made for revertants of two of these mutants, P1 and C31, from *frd*^{ts} to *frd*$^{+}$. Starting with single-plaque isolates of P1 and C31 progeny, phage particles were examined for their ability to form normal plaques, i.e., without white halos, at the high temperature (37°C). Two revertants each of P1 and C31 were found, and the plating properties of the revertants are given in Table 2. All the revertants isolated were still resistant to the addition of pyrimethamine to the plating media, and it can be concluded that the change in temperature sensitivity of the free dihydrofolate reductase, as shown by the plaque assays, was due to a second mutation independent of the site sensitive to pyrimethamine.

The heat sensitivities of the particles produced by the two revertants of P1 are shown in Fig. 3, and the heat sensitivities of the two C31 revertant particles are shown in Fig. 4. One of the P1 *ts* revertants had the same sensitivity as the parent P1, whereas the other revertant was more heat resistant. Both of the *ts* revertants of C31 produced phage particles with heat sensitivities different from the parent C31 but in this case the revertants produced particles that were more heat sensitive than the parent C31. These properties support the conclusion that dihydrofolate reductase is a phage baseplate component and indicate that any change in the polypeptide (such as to greater heat resistance of free enzyme) may change the heat sensitivity of the baseplate to either greater heat resistance, such as the P1a revertant, or more heat sensitivity, such as the C31a or C31b revertants.

TABLE 2. *Plating properties of various T4D frd^{ts} revertants to T4D frd^{+}*

Phage strain	Plaques on OK305 at:		Plating efficiency on OK305 + (P+S)[a] at 37°C
	37°C	32°C	
$T4D_0$	No halo	No halo	0.02
P1	Halo	No halo	1.06
Revertant a	No halo	No halo	0.70
Revertant b	No halo	No halo	1.03
C31	Halo	No halo	0.98
Revertant a	No halo	No halo	1.02
Revertant b	No halo	No halo	0.92

[a] Similar to the medium used by Johnson and Hall (9) but containing lower concentrations of pyrimethamine (P) and sulfanilamide (S); final P = 70 μg/ml, and final S = 4.5 μg/ml. The plating efficiency is the ratio of plaques on OK305 + (P+S) to the plaques formed on OK305 without (P+S).

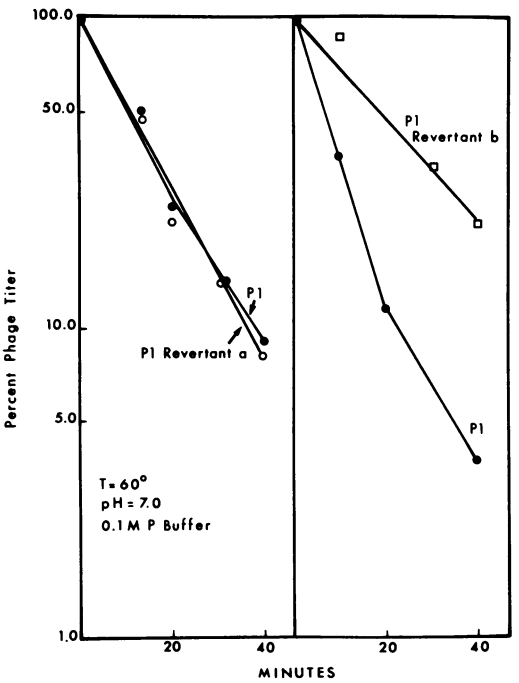

FIG. 3. *Heat sensitivity of T4D P1 frd^{ts} and P1 frd^{+} revertant particles. Two revertants of P1 from frd^{ts} to frd^{+} were isolated as described in Table 2. Phage stocks were grown at 37°C, and the heat sensitivities at 60°C were determined individually in the usual way. P, Phosphate.*

Properties of phage bearing deletions between genes 63 and 32. Homyk and Weil (7) have described the isolation of three viable T4 strains presumably bearing long deletions of the region between genes 63 and 32 on the T4 linkage map, which would include the gene for *td* (*del*1) and both genes *td* and *frd* for both *del*7 and *del*9. Whereas the enzymological activities of infected extracts (3) support the view that these genes do not produce the appropriate functional enzymes, other properties suggest that the heteroduplex physical mapping of the DNA is sufficiently inaccurate so that the formation of *td* and *frd* gene-related polypeptides cannot be excluded. The problems of interpreting the nature of these deletions are given in detail in the companion paper by Mosher et al. (22) and are summarized only briefly here.

(i) Immunological studies have shown not only that these deletion mutants produce cross-reacting material to T4 phage thymidylate synthetase and T4 phage dihydrofolate reductase, but also that the phage particles are even more susceptible to inactivation by antisera to these enzymes (22) than is T4D. Whereas the sensitivity of *del*1 to anti-*frd* serum is not surprising, both *del*7 and *del*9, which presumably lack the *frd* gene, are even more sensitive to this antiserum than is *del*1 or parent T4D. (ii) Furthermore, genetic experiments by Mosher et al. have shown that the *frd* gene is not deleted in *del*7 or *del*9, but that the enzymatic activity is probably suppressed by some other, additional mutation. (iii) A replotting of the deletions on the T4 map (22) shows that these deletions must lie 2,000 nucleotide pairs more clockwise (to the left) and, therefore, would not delete the *frd* gene and probably not the *td* gene.

Additional chemical and physical properties of these deletion mutants were characterized and are summarized in Fig. 5. When the deletion mutants, *del*1, *del*7, and *del*9, are treated with pyridine nucleotides, such as NADPH and NADH, they are inactivated. The inactivation reaction has been attributed to an interaction with a pyridine nucleotide binding site on the phage baseplate (16). It should be noted that NADPH inactivated these particles somewhat more rapidly than NADH, in accord with the properties of the dihydrofolate reductase molecule. The phage-binding site was also shown to be specific for one of the two isomers of the pyridine nucleotides (16) and to include a bind-

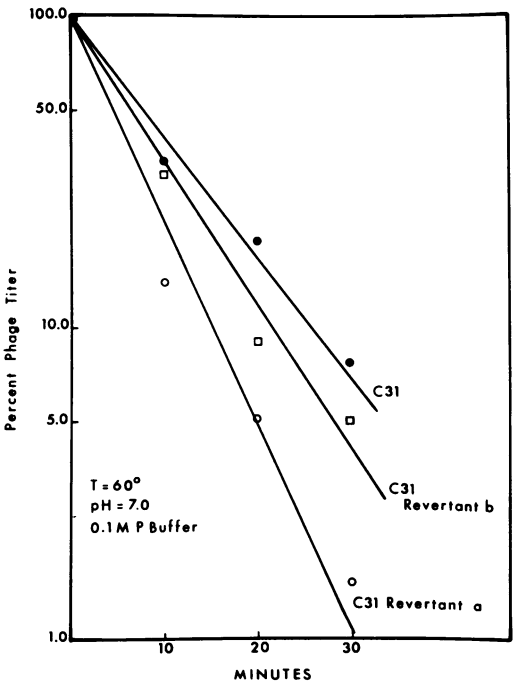

FIG. 4. *Heat sensitivity of T4D C31 frd^{ts} and C31 frd^{+} revertant particles. Two revertants of C31 from frd^{ts} to frd^{+} were isolated as described in Table 2. Phage stocks were grown at 37°C, and the heat sensitivities at 60°C of the parent C31 and the two revertants were determined in the same experiment. P, Phosphate.*

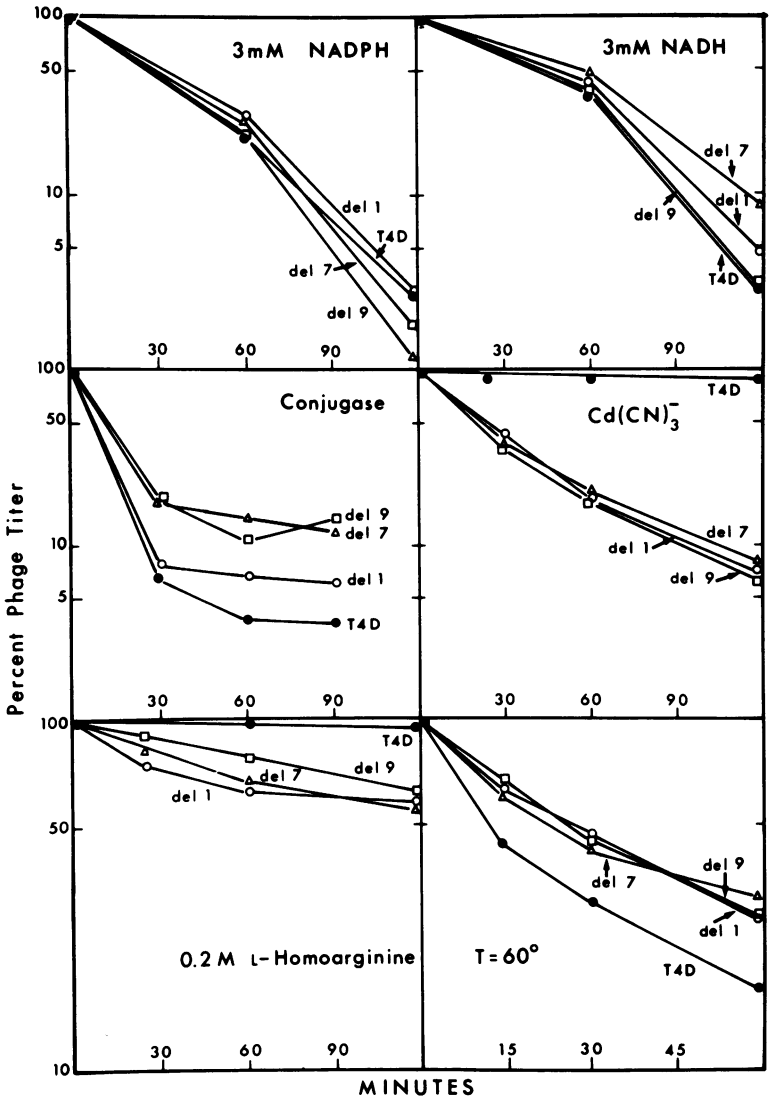

FIG. 5. *Effect of various inactivation treatments on different T4D strains. Stocks of the parent T4D strain 1589 and the three deletion mutants of Homyk and Weil were prepared and subjected to various treatments that primarily affect the baseplate structure. Details of these procedures are given earlier for NADPH and NADH (12), conjugase (14), $Cd(CN)_3^-$ (13), and L-homoarginine (23), and the heat treatment was the standard procedure used in this work.*

ing site for the adenosine diphosphoribose portion of the nucleotide (19). Furthermore, it was found that hog kidney conjugase, an enzyme specific for hydrolyzing the γ-glutamyl bonds of folate polyglutamates, also inactivated these particles (14). In several experiments, it was noted that the strain T4D 1589 (into which these deletions were crossed) was always inactivated more rapidly and to a greater extent than the three deletion mutants. These properties show that the three deletion mutants have a tail structure somewhat different than T4D 1589 but that they still contain the phage folate and enough of the dihydrofolate reductase molecule so that the phage particles are sensitive to pyridine nucleotides.

Three other properties of the baseplates of these three deletion mutants as compared to those of the T4D strain 1589 are also shown in Fig. 5. All three deletion mutants were readily inactivated by the reagent $Cd(CN)_3^-$ (13) or by incubation with L-homoarginine (23), whereas

T4D strain 1589 was highly resistant to these two reagents. Further, T4D strain 1589 was more readily inactivated by heating at 60°C than were the three deletion mutants. Since $Cd(CN)_3^-$, L-homoarginine, and heating at 60°C (12, 15) all primarily perturb the baseplate, these results confirm the observations that the physical properties of the baseplates of the deletion mutants are different from those of the T4D 1589 strain. However, deletions of the phage genomes between genes 63 and 32 have not been thought to include sequences for any essential phage structural components (25). These results indicate that these deletions do affect structural components. The identification of the structural components in these mutants as polypeptides related to thymidylate synthetase and dihydrofolate reductase molecules rests on the immunological, enzymological, and genetic (22) data, the inactivation of the phages by pyridine nucleotides, the presence of phage folic acid, and the restriction of these strains in certain hospital strains of *E. coli* (22).

Properties of deletion mutants grown on various strains of *E. coli*. Various *E. coli* strains were available with different amounts or types of host dihydrofolate reductases. RT500, an *E. coli* B strain resistant to high levels of trimethoprim, has been shown by Baccanari et al. (1) to overproduce the normal *E. coli* B *frd*. We isolated earlier several *E. coli* B mutants that were also resistant to trimethoprim (12) and showed that the dihydrofolate reductases produced in these mutants were significantly altered, as judged by their heat sensitivity. These bacterial strains offered an opportunity to compare the deletion mutant particles assembled in different cells. Since *del*7 and *del*9 are presumed to lack the T4D *frd* gene, whereas *del*1 does not lack this gene, any substitution of host enzymes during viral morphogenesis would be expected to alter the heat sensitivity of *del*7 and *del*9 phage particles, as compared with either *del*1 or T4D 1589. The three deletion mutants and T4D strain 1589 were grown on *E. coli* CR63, *E. coli* B RT500, and the *E. coli* B TRIM 102 and TRIM 103 (12) mutants. It was found that the T4D strain 1589 produced in all these host cells was somewhat more heat sensitive than the three mutants (see Fig. 5). But all three mutants, *del*1, *del*7, and *del*9, gave very similar heat sensitivities to each other, irrespective of the host cells upon which they were grown (data not shown). In addition, since these particles are also sensitive to antisera to T4D dihydrofolate reductase and do react with the co-factors for this enzyme, NADPH and NADH, it can be assumed that all three deletion mutants do not, in fact, lack the ability to synthesize a protein similar to the original T4D dihydrofolate reductase.

DISCUSSION

The data in this paper and the accompanying paper by Mosher et al. (22) provide confirmation that both the T4D *frd* and the T4D *td* genes produce components that are normal baseplate components of the T-even bacteriophages. Furthermore, these papers support the earlier views that these enzymes are partially buried within the complex tail structure. It is not surprising that, whereas phage ghost preparations do exhibit both detectable dihydrofolate reductase and detectable thymidylate synthetase activities, which have been identified as being due to phage gene products, the activities are extremely low. The low activities are, most likely, due to the occlusion of both enzymes in the phage structure and the close affinity of the folate, which would prevent most of these enzyme molecules from reacting catalytically. The data also suggest that these proteins need not be complete polypeptides to fulfill their structural role, since polypeptide fragments produced by amber mutants in the *frd* or *td* gene can participate in phage assembly (12).

The quantitation of these two polypeptides in the baseplate remains a difficult problem, and Mosher et al. (22) have put an upper limit of about six molecules of each per phage particle as the sensitivity limit of their analytical procedure. This value would be in accord with a similar value for the six folates per phage particle (14, 15).

The question of whether the two enzymes are essential or dispensable gene products for assembling the baseplate is not completely resolved by these experiments. The evidence for concluding that they are essential components of the baseplate rests on the following data. (i) They have been detected directly or, in some cases, indirectly by some significant chemical or genetic property in all T4 strains so far analyzed, with the only possible exception being some partially characterized T4D deletion strains. The properties of the deletion mutants, which are analyzed in detail for the presence of *frd* by Mosher et al. in the companion paper (22) and for the presence of *td* in this paper, suggest that these deletion mutants do contain *frd*- and *td*-like polypeptides. Earlier observations with the missense and nonsense mutations support the view that these enzymes need not be functional for them to play a structural role. (ii) The evidence on the heat sensitivity of various phage frd^{ts} and td^{ts} mutants and their revertants, coupled with the confirmation by

Mosher et al. (22) that the baseplate is the most heat-sensitive component of the virus particle, support the idea that these two proteins are integral parts of the baseplates and not adventitious constituents attached to the outside of the baseplate. (iii) The question remains whether there could be phage particles that were completely infectious but that lacked these two components. One analogy would be to the *hoc* and *soc* proteins, which have been recently shown by Ishii and Yanagida (8) to be normal but dispensable components of the T4 phage head. It should be noted that, whereas Ishii and Yanagida (8) readily isolated T4D mutants lacking the *hoc* and *soc* components, the situation, at least with regard to phage *frd*, is different. A search was made for a T4D mutant (19) resistant to inactivation by the *frd* cofactor NADPH, and no mutants were obtained from 10^{12} T4D particles. This means either that the *frd* polypeptide with a binding site for NADPH is absolutely required or that a T4D mutation lacking *frd* is extremely rare. There is no comparable method for searching for the absence of the phage *td* in phage mutants. Nonetheless, since the conservation of a gene product for both structural and metabolic roles is unusual, the possibility must still be considered that one or both of these unexpected phage components are dispensable.

ACKNOWLEDGMENTS

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